The Development of Thermoreversible Progesterone-Loaded Hydrogels for the Prevention of Preterm Birth

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Abstract

Preterm birth (PTB) (<37 weeks of gestation) is the second largest direct cause of mortality in children under the age of five. Infants born preterm often have various medical complications, including neurological disorders, leading to increased morbidity. PTB is multifactorial and few efficacious therapies exist. Currently, the only therapeutic that has shown efficacy against PTB is progesterone. Vaginal drug delivery is the preferred route for targeting the female reproductive tract. With effective localized delivery, vaginally delivered drugs have a high permeation range, avoid first pass metabolism, and experience lower enzymatic activity, which leads to an increased drug concentration in local tissues. However, key barriers to effective vaginal drug delivery, including the protective cervicovaginal mucus (CVM) that acts as a barrier and facilitates clearance, must be considered. Clinically, progesterone is often dosed vaginally for the prevention of preterm birth (PTB). Crinone® is a hypertonic gel containing micronized progesterone used for this purpose. The prevention of PTB with Crinone® has varied efficacy, likely due to its formulation which is hypertonic and micronized. Here, I describe a novel *in-situ* hydrogel for vaginal progesterone delivery that demonstrates more superior physical properties in terms of particle size, osmolality, surface charge and viscoelastic profile when compared to Crinone®.

Previous studies have demonstrated that non-adhesive mucus penetrating particles (MPP) (with hydrophilic arms and a hydrophobic core) can effectively overcome the CVM to deliver drug loads to the underlying epithelium. However, due to liquid formulation MPP alone is not the most desirable mode of delivery. Here, I tested whether an *in-situ* hydrogel composed of progesterone loaded MPP could serve as a more effect mode of drug delivery. I hypothesized that Pluronic F127, a nonionic amphiphilic triblock copolymer with thermoreversible properties based on concentration, could create an *in-situ* gel upon vaginal delivery. Nanoparticles were developed using various Pluronics and the most desirable nanoparticle formulation was selected based size, ζ-potential, polydispersity and tonicity. Separately, various F127 gels were characterized for rheological properties such as sol-gel transition. Finally, nanoparticles and gels were combined and further tested for physical characteristics and in vivo properties. I found that nanoparticles with lower concentrations milled at higher volumes with ZsOBO5 beads with low concentrations of stabilizer had the smallest size, PDI, and ζ-potential, as desired. F127 gels from

14-18% w/w demonstrated the most desirable physical properties based on rheological and *in vivo* tests. Of all the hydrogels tested the 16% w/w gel with 2% F127 and 2% F68 coated NC had the greatest stability.

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List of Abbreviations

CP	Conventional particles (PS
CVM	Cervicovaginal mucus
MPP	Mucus penetrating particle
P-gp	P-glycoprotein
PS	Polysterene
PSPEG	Polysterene poly(ethylene glycol)
PTB	Preterm birth
PEG	Polyethylene glycol
HSV	Herpes simplex virus
HIV	Human immunodeficiency virus
MSD	Mean square displacement
MPT	Multiple particle tracking
PEO	Poly ethylene oxide
PPO	Poly propylene oxide
ZsO05	Zirconium silicate 0.5 mm
ZrOB015	Zirconium oxide 0.15 mm
ZrOB05	Zirconium oxide 0.5 mm
PDI	Polydispersity index
NS	Nanosuspension
NC	Nanocrystal
NP	Nanoparticle
DI	Deionized
TBS	Tris buffered saline
G’	Storage modulus (elastic component)
G”	Loss modulus (viscous component)
γ	Strain
γ_c	Critical strain
δ	Phase shift
τ	Shear stress
η	Viscosity
ν	Velocity
Đ	Dispersity
1.16	2% F127 nanoparticle in 16% F127 gel
2.16	2% F127 with 2% HS15 nanoparticle in 16% F127 gel
3.16	2% F127 with 2% F68 nanoparticle in 16% F127 gel
4.16	2% F127 with 2% TPGS nanoparticle in 16% F127 gel
5.16	4% F127 nanoparticle in 18% F127 gel
1.18	2% F127 nanoparticle in 18% F127 gel
2.18	2% F127 with 2% HS15 nanoparticle in 18% F127 gel
3.18	2% F127 with 2% F68 nanoparticle in 18% F127 gel
4.18	2% F127 with 2% TPGS nanoparticle in 18% F127 gel
5.18	4% F127 nanoparticle in 18% F127 gel

Introduction and Background

The Significance of Preterm Birth

The World Health Organization estimates that every year approximately 15 million babies are born prematurely, the highest rates occur in Africa and North America. One million of those babies die from complications related to preterm delivery [1]. Preterm birth (PTB) is the leading cause of mortality and morbidity in children under the age of five. PTB can cause brain injury via neuroinflammation resulting in a cascade of central nervous system responses which causes damage to the developing brain [2-3]. In 2016, PTB affected approximately 10% of infants in the United States [4]. Not only does PTB have significant societal ramifications, the medical cost of associated with care exceeds $25 billion per annum [5]. While there are multiple drugs on the market to prevent PTB, including the only FDA approved drug solely designated for this purpose, hydroxyprogesterone caproate (Makena); the main vaginal delivery drug, Crinone®, has low and varied efficacy ranging from 33.1% to up to 58.1% in clinical pregnancies [6]. Therefore, it is imperative that we find a tolerable and efficacious alternative to address this problem. While the causes of PTB vary and some of them are not fully understood, we know that the progesterone inflammation pathway plays a significant role in causing preterm labor.

Physiological Challenges Associated with Localized Vaginal Delivery

The Role of Cervicovaginal Mucus in the Context of Vaginal Delivery

The mode of administration is also of critical importance when treating PTB. Vaginal delivery offers many advantages over other routes of administration: effective localized delivery, huge permeation range, large vascularization, avoidance of first pass metabolism and lower enzymatic activity [7]. The female reproductive tract (FRT) is covered in cervicovaginal mucus (CVM) which makes vaginal delivery particularly challenging. A clear understanding of vaginal physiology and anatomy is essential for the development of localized drug delivery platforms. The mucus is a viscoelastic gel layer that protects tissues from the external environment. The CVM is composed of a mesh of interwoven mucin fibers, proteins, cells, lipids and bacteria [8]. Each of the components works together to form a nanoscopic heterogenous environment that functions as the critical barrier against pathogens. Typically, there are steric interactions as well

as the physical restrictions of pores that are a challenge for local delivery of xenobiotics. The CVM has an average pore size of 340nm +/- 70nm, desirable nanoparticles would have a smaller hydrodynamic diameter [8]. In previous mucus studies conducted at the Center for Nanomedicine (CNM), we have seen as pregnancy progress the size of the pores decreases. Additionally, during pregnancy the mucus thickens forming a plug that protects the uterus and the growing fetus changing the physical landscape of the CVM [9]. Factors such as infections/diseases and physical stressors can also affect the microbiome of the CVM leading to a reduction in barrier properties [8].

CVM plays a significance role in the female reproductive tract (FRT) by acting as a constant barrier against foreign pathogens and rapidly clearing any particulates from the system. Forty percent of preterm births are caused by some form of intrauterine infection and typically results in a gestation of 32 weeks or less [10]. In such cases a microbial infection reaches the amniotic cavity causing PTB. In healthy patients, high progesterone levels during pregnancy lead to a strengthening of the mucus plug which covers the cervix to prevent pathogens and infections from reaching the uterus. However, when the mucus barrier is comprised by mechanisms such as those involved in bacterial vaginosis, the impaired CVM may lead to increased risk of PTB [11]. Thus, when developing a therapy for PTB we must consider the role of mucosal barrier and delivery through the mucosal pores.

The Role of Epithelial Lining for Drug Delivery

The vaginal epithelium is composed of distinct layers of apical, lateral and basal plasma membranes. Epithelial polarity has two functional directions from apical into the basolateral region or basolateral to apical permeability. The direction of polarity is impacted by multiple factors, including P-gp substrate mediated transport, that increases apical to basolateral permeability and decreases basolateral to apical permeability. Mucosal epithelia also rely heavily on osmotic gradients to mediate transport. Osmotic driven secretions of vaginal products have been shown to have a rate proportional to the hypertonicity of the given formulation *in vivo*. Secretions not only cause immediate loss of the product but also produce local inflammation and cause discomfort in patients. Therefore, fluid uptake and secretion can be controlled through

changes in tonicity of the vaginally delivered product. The Ensign lab has previously demonstrated that hypotonic formulations of nanoparticles (MPP) increase fluid absorption and thus causing convective transport of drug loaded nanoparticles to the vaginal epithelium [12].

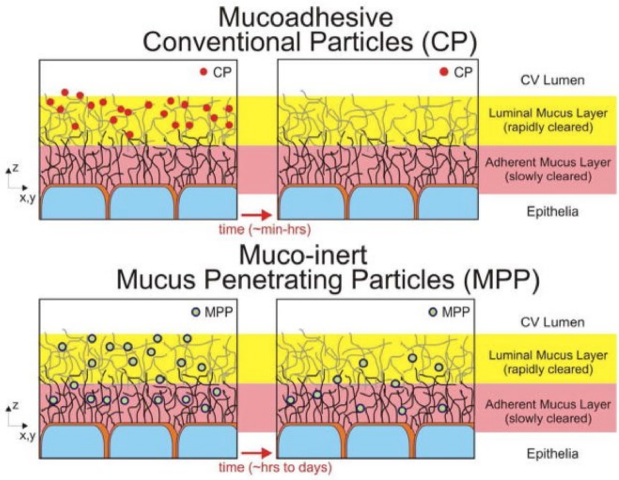


Figure 1. Polystyrene coated conventional nanoparticles are trapped in the luminal mucosal layer due to steric hinderances and are rapidly cleared. Comparatively, muco-inert Pluronic or polyethylene glycol coated mucus penetrating particles have a greater diameter but are able to diffuse through both mucosal layers to reach the epithelia lining and release the drug load

As previously described, MPPs rapidly diffuse through mucosal barriers to reach the underlying squamous epithelial cells. Comparatively, conventional particles (CPs) are trapped in vaginal mucus. CPs are trapped due to steric hinderance and interactions with hydrophobic particles and hydrophobic regions within mucin fibers. As previously reported by the Ensign lab, MPPs have a muco-inert coating that allow particles up to 500nm in diameter to penetrate the CVM. Nanoparticles and other xenobiotics that cannot diffuse through the mucus layer are trapped within mucus and cleared between minutes and up to hours as seen in Figure 1 A. Effective delivery of NP gel within the CV tract requires the delivery system to penetrate the mucosal barrier without rapid clearance. This problem could be mitigated through a thermoreversible gel with desirable sol-gel properties would also be advantageous because it forms *in situ* allowing for higher retention.

Characteristically mucus is shear thinning and has a rapidly clearing top layer over a slowly cleared adherent layer of mucus above the apical level of epithelial cells. Therefore, particles

need to diffuse quickly through to penetrate rapidly cleared mucus layer to reach the underlying adherent mucus layer. Loaded MPPs can rapidly diffuse through the CVM to reach the epithelia and provide sustained release of their payload. MPP are specifically formulated to penetrate the mucosal barriers due to their high density and low molecular weight polyethylene glycol coating. PEG is uncharged and thus muco-inert which subsequently avoids steric and ionic interactions. The MPP does not adhere to hydrophobic and charged regions of mucin allowing PEG coated MPP to slip through pores in the CVM. Although the PEG coating increases the hydrodynamic diameter, the neutral surfaces mitigate "trapping" effects of mucus. Other diblock co-polymers, Pluronics, are commonly used for covalent linkages and coatings in MPP.

Current Therapies and the Associated Challenges

Currently, the only FDA approved therapeutic active ingredients that has shown efficacy against PTB are progesterone and its synthetic analogs [13]. Progesterone plays a natural role in the establishment and maintenance of pregnancy [14]. The pharmacological action of progesterone (P4) is to inhibit cervical ripening and prevent delivery. In humans, functional progesterone withdrawal signals the end of pregnancy [15]. It has been theorized that progesterone supplementation could lead to a more anti-inflammatory state and consequently prevent PTB. A successful drug candidate would be able to reduce the inflammation associated with PTB.

Conventional vaginal gel products consist of particles that are 1 micron or larger in size making it difficult for them to pass through the pores of the CVM and thus rendering them ineffective. Crinone®, a micronized progesterone gel, is currently approved for vaginal administration and has been used in clinical studies for pre-term birth [16-17, 6]. During a randomized, double blind placebo-controlled trial daily dosing of Crinone® reduced the rate of PTB before 33 weeks by only 44% [16]. Furthermore, vaginal gel products are typically hypertonic, causing fluid secretions and subsequently rapid clearance of drug from the vagina. Using Crinone® many women have reported discharge and discomfort, consequences of localized toxicity and inflammation, which counteracts the anti-inflammatory properties many researchers aim to prove with progesterone formulations [18]. The negative effects associated with hypertonic gels can

also increase the risk of bacterial vaginosis (BV) and other infections, which are known risk factors for preterm labor.

The Use of Pluronics and Polymeric Stabilizers

With topically delivered formulations drug failure in clinic is typically due to the hydrophobicity and low bioavailability of drug candidates. Nanocarriers offer a method of encapsulation that manipulates hydrophobic drugs and confines them into nanoscale particles which can be used in a stable homogenous suspension. Thus, allowing for more applicable clinical use. Poloxamers or Pluronics have been used in the biomedical field for a wide variety of applications including by not limited to emulsifiers, additives, surfactants and permeation enhancers. Pluronics are a group of synthetic nonionic block copolymers composed of a central chain of hydrophobic poly(propylene oxide) flanked by two hydrophilic chains of poly(ethylene oxide). Each Pluronic has a A-B-A triblock structure consequently giving them an amphiphilic structure as seen in Figure 2. Each Pluronic triblock polymer is named by letter and a proceeding two or three digits. The letter refers to the physical form of the copolymer at room temperature (F for flake, P for paste and L for liquid). While the first number corresponds to the PPO molecular weight and the last number represents the percentage of PEO molecular weight of the total molecular weight.

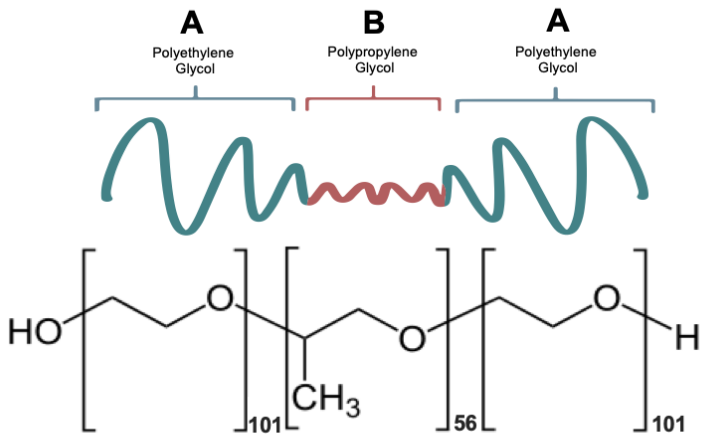


Figure 2. Chemical structure of Pluronic F127, composed in an A-B-A co-block structure with repeat units of PEO (A) and PEG (B).

Pluronics for the Creation of Nanoparticles

A common Pluronic, Pluronic F127, is hydrophilic coblock polymer of PEO (100)- PPO (65)-PEO (100). Due to F127's amphiphilic characteristics they possess surfactant properties which allow them to interact with hydrophobic surfaces as well as biological membranes [19]. Additionally, being amphiphilic allows Pluronic to combine and form micelles, with a hydrophobic core and a hydrophilic shell, in aqueous solutions. Pluronic mixed in an aqueous solution with drug can act as a surfactant, coating and loading the drug into the hydrophobic core, thus creating nanoparticles [20]. Properties such as particle stability (measured through zeta potential) and hydrodynamic diameter can be optimized through the use of specific Pluronics and stabilizers as well as conditions like type of mixing (wet milling vs high pressure homogenization) and mixing time. For our purposes a variety of Pluronics and stabilizers were used, while mixing conditions were unchanged.

Pluronic F68 is a non-ionic surfactant that is frequently used to control shear forces in suspensions. The difunctional co-block polymer has been previously been used to increase pore formation and optimize drug releasing character of nanosuspensions. Pluronics generally have desirable biological activity; specifically, they are able to incorporate into membranes, translocate into cells and subsequently effect various cellular functions including mitochondrial respiration and ATP synthesis. Both of which have been studied extensively for absorption and drug interactions. F68 inhibits P-gp and CYP3A4 which leads to a reduction in metabolism hence decreasing drug interactions as described by Cornaire and Gonzalez and decreasing variability of the absorption site [21-22].

d-alpha-tocopheryl poly (ethylene glycol) 1000 succinate (TPGS) is a safe pharmaceutical adjuvant. TPGS is a water-soluble derivative of vitamin E that is commonly used in the pharmaceutical and nutraceutical industries to enhance the bioavailability of poorly soluble active molecules. TPGS offers the benefits of both PEG and vitamin E in various nanocarriers for drug delivery including extended half life and enhanced cellular uptake. Additionally, the amphiphilic structure, a lipophilic alkyl tail and a hydrophobic polar head allows for a low critical micelle concentration of 0.02% w/w [23]. Previous studies have reported that TPGS is ideal for prodrug, micellular, liposomal and nanoparticle delivery with sustained, controlled and targeted applications. Across industry and research, TPGS has been used as an absorption

enhancer, additive, solubilizer, emulsifier, permeation enhancer and stabilizer. Feng et al describes a TPGS based nanoparticle copolymer system loaded with taxol or doxorubicin that increased uptake efficiency in Caco-2, HT-29, MCF-7 and C6 glioma cells. Furthermore, when used as a surfactant, TPGS was used to created emulsified nanospheres along with PLGA. As an additive for nanoparticles both encapsulation efficiency (EE) and sustained release were increased. Encapsulation efficiency increased from 69.0% to 75.9% with 4.2 drug loaded with the addition of 5% TPGS and 90.1% EE was achieved with 2% drug and 5% TPGS [23-24].

Additionally, TPGS and some other co-block have been shown to inhibit P-glycoprotein (P-gp) activity. P-gp is found primarily in the apical surface of epithelial cells and as part of the ATP-binding cassette (ABC) transporters it acts as a physiological barrier by binding therapeutics substrates. P-gp is responsible for efflux membrane transfer by limiting cellular uptake and distribution of xenobiotics and thus mediating drug-drug interactions [25].

Additionally, I utilized HS 15 which is a potent non-ionic solubilizer and emulsifying agent with low toxicity. HS 15 is a low molecular weight agent used in the manufacture of aqueous parenteral formulations that is composed of polyglycol mono and lipophilic dierters of 12 hydroxystearic acid and 30% hydrophilic free PEG. HS 15 has a high solubilization capacity, low viscosity, thermos stability, good biocompatibility and desirable micellular properties.

Pluronic as a Hydrogel Platform

Additionally, concentrated solutions of Pluronic have a thermoreversible gelation property: they are able to transition, in a certain temperature region, from a viscous liquid to an assembly of effectively crosslinked molecules while maintaining elasticity. The sol-gel transition temperature is governed by concentration, molecular weight and composition of each block polymer [26]. Therefore, thermosensitive Pluronic hydrogels have attracted significant interest in the scientific community and have been used for a variety of applications ranging including food products, drug delivery and tissue engineering.

Despite the frequent use of thermoreversible hydrogels in pharmaceutics and medicine, few studies have utilized in situ hydrogels for vaginal delivery. Furthermore, I investigated the use of mucus penetrating particles in combination with a hydrogel.

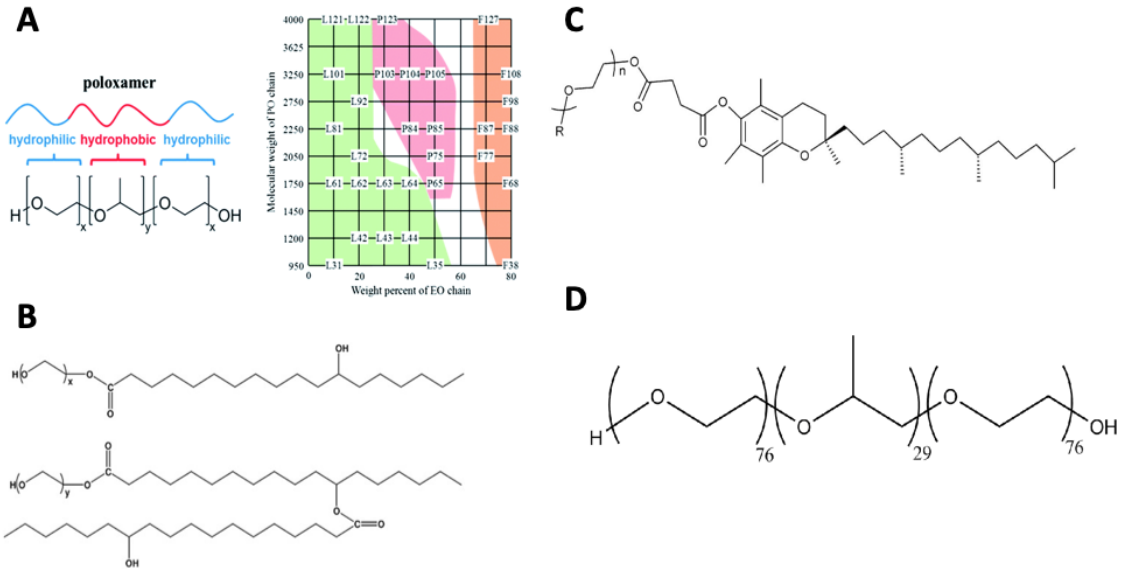


Figure 3. A. General PEO-PPO-PEO amphiphilic structure of Pluronics and the weights of various Pluronics. Chemical structures of excipients [27] B. amphiphilic copolymer excipient Kolliphor HS15 [28] C. TPGS as Vitamin E [29], and D. Pluronic F68 [30].

Nanoparticle Development

Introduction

The CVM's mesh of mucin proteins forms a hydrophobic and charged net to sterically hinder foreign entities. The CNM has previously characterized the barrier properties of the CVM in healthy, non-pregnant women and shown that trapping occurs with conventional particles greater than 100nm. Furthermore, our collaborator, Dr. Richard Cone, has demonstrated viruses and pathogens can diffuse through the mucosal layer rapidly, indicating that the surface chemistry also impacts mobility in the CVM [31]. Therefore, in order to design an effective mucus penetrating drug platform it must have specific properties including size characteristics and surface chemistries that facilitate diffusion through the CVM. This led us to the design non-adhesive mucus penetrating particles (MPPs) (with hydrophilic arms and a hydrophobic core). I was then able to investigate the effect of MPPs and fluorescently labelled virions in samples of CVM from healthy subjects. I used an imaging technique that allowed us to track hundreds of particles in a sample called multiple particle tracking (MPT). Using this method, I determined that CVM mesh pores were larger than previously published data had suggested [32]. MPPs as large as 500nm could diffuse through mucus. Further investigation showed us that healthy vaginal mucus adhesively traps herpes simplex virus (HSV) and human immunodeficiency virus (HIV), which helps to explain why health vaginal microbiota reduces the risk of sexually transmitted infections [20]. Based on these findings I could sufficiently quantify the structural and barrier properties of the CVM in hormonal and diseased states. Our group at the CNM used the average mean square displacements (MSD) as a function of time to illustrate the diffusion of nanoparticles and HIV over time. MSD is calculated by transforming the coordinates of nanoparticle centroids into time averaged MSD.

$$< \Delta r^2(\tau) > = [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2 \quad \text{.....Equation 1}$$

where x and y represent the 2D nanoparticle coordinates at a given time and t is the time scale [33]. Previously the static error has been estimated to 20 nm, and we assume that time scale of 1 s is relatively long to the interval between frames to reduce the contributions of dynamic error [12, 33-34]. As shown in Figure 4, 100nm MPP can diffuse through the CVM of both healthy and BV women at similar rates [35]. Conventional particles (CP) of 100 nm, in comparison, diffuse much more slowly in healthy mucus, while BV secretions have reduced adhesive

trapping of CP. Thus, particles below 500 nm and ideally, in a range of 200 nm to 100 nm have the most desirable size characteristics.

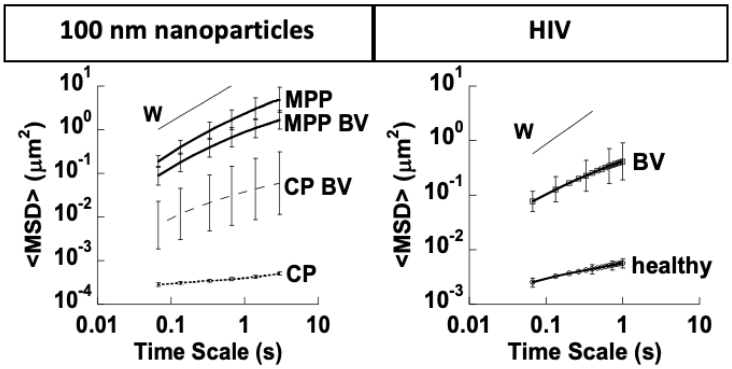


Figure 4. Ensemble-averaged geometric mean square displacements (<MSD>) as a function of time scale in cervicovaginal mucus (CVM) from women with healthy vaginal microbiota and women with bacterial vaginosis (BV). A reduction in adhesivity was seen in BV secretions, as evidenced by the diffusion of CP. Reduced adhesion and diffusion of HIV was also observed in BV secretions compared to immobilization of HIV in CVM from women with healthy microbiota (healthy). W = MPP/HIV diffusion rate in water [35].

Methods

MPP were prepared using a previously reported method on wet milling nanocrystal synthesis using a bead-based tissue homogenizer by Hoang et al [36]. Initial optimizations included varying bead type, F127 concentration, the inclusion of additional stabilizers and the total volume of solution. Briefly, various progesterone MPPs were made at concentrations of 2% w/w, 4% w/w and 6% w/w F127 alone or with 2% TPGS, 2% F68 or 2% HS15 all obtained from BASF. This totaled to 90 unique combinations of progesterone nanoparticles.

Zirconium beads were used as agitators in the milling process. Zirconium silicate (ZsOB5, 0.5 mm diameter) and Zirconium oxide beads (ZrOB05, 0.5 mm diameter and ZrOB015, 0.15 mm diameter) from NextAdvance were loaded into 2 mL, 1.5 mL and 0.5 mL Eppendorf tubes for a total mass of 2000 mg, 1500 mg and 1000 mg, respectively. Pluronic F127, P4 and the additional stabilizer were loaded into tubes. Based on the tube size, 1.5 mL, 1.0 mL or 0.5 mL of deionized water was added to suspend the mixture. The solution was kept at 4°C overnight and subsequently wet milled for a total of ten hours. Every 2 h size and polydispersity index (PDI)

measurements were taken. At 10 h ζ-potential was measured. The nanosuspensions were kept at 4°C and stability measurements (ζ-potentials, size, PDI) were taken at 24 h, 48 h, 72 h and 168 h.

Both the hydrodynamic diameters and ζ-potentials of the nanoparticle formulations were characterized by dynamic light scattering and laser Doppler anemometry, respectively. The sizes of the NP were determined using Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA). Size measurements were performed at 25°C at a scattering angle of 90°. To determine the ζ-potential particles were diluted in a 10mM NaCl solution diluted from Dulbecco's phosphate-buffered saline (Cellgro, Mediatech, Inc., Manassas, VA) at pH 7. ζ-potentials were measured by laser Doppler anemometry using Zetasizer Nano ZS. All measurements were performed in accordance to instrument instructions.

Osmolality was measured using Wescor EliTechGroup Vapor vapor pressure Osmometer. The device was calibrated with 100, 290 and 1000 mOsm/kg solutions. After calibration, 10 μL of undiluted NS was measured.

Results and Discussion

The hydrodynamic diameter and PDI of the 90 formulations were measured and the mean size was determined at all time points. Formulations with that maintained a size below 350 nm from 10 h to 168 h were selected as potential candidates. Nanosuspensions that consisted of 2% w/w F127 either with stabilizer or without typically had a smaller diameter ranging from 233-380 nm, in comparison to >388 nm diameters of formulations with 4% w/w F127 and 6% w/w F127. Additionally, 2% w/w F127 nanosuspensions made with the ZsOB5 beads had smaller diameters when compared to formulations milled with ZrOB05 and ZrOB015 beads. PDI was measured to determine the heterogeneity of the given NC samples. NC with the PDI Đ < 200 were further selected from the 90 formulations.

The ζ-potentials at 10 h, 24 h, 48 h, 72 h and 168 h were found for all formulations. The nanosuspensions that demonstrated the most neutral net charge were selected from the list of NS with acceptable particle diameter. A near neutral ζ-potential was used to confirm PEG conjugation.

With size, PDI and ζ-potentials the "top five" NS could be found as seen in Table 1. Additionally, the osmolality of these formulations was measured to confirm their tonicity. Osmolality of all of the selected nanosuspensions were hypotonic. As previously reported hypotonic solutions enhances vaginal delivery by increasing fluid uptake into the vaginal epithelium [37].

Formulation	**Size (nm)**	**ζ-potential (mV)**	**Osmolality (mOsm/kg)**
NC 2% F127	297 ± 16	-1.81 ± 0.15	7 ± 1.53
NC 2% F127 + 2% TPGS	320 ± 11	-1.48 ± 0.55	11 ± 3.00
NC 2% F127 + 2% F68	257 ± 12	-1.04 ± 0.26	18 ± 2.08
NC 2% F127 + 2% HS15	322 ± 18	-1.13 ± 0.74	19 ± 1.15
NC 4% F127	229 ± 4	-0.762 ± 0.63	12 ± 11.93

Table 1: Physical properties of selected progesterone nanoformulations. After milling the progesterone loaded NC coated with either 2% w/w F127, 2% w/w F127 and F68 and 4% w/w F127 demonstrated desired characteristics of NC.

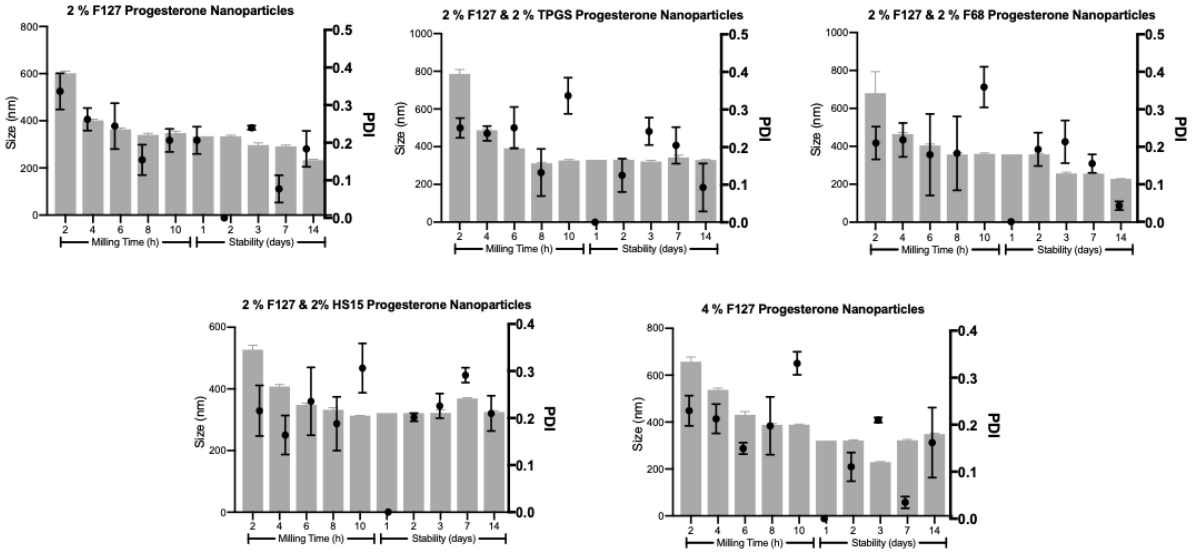


Figure 5. Diameter, polydispersity index (PDI) and **ζ**-potential at 2 h timepoints over the course wet milling and then at 24 h, 48 h, 72 h, 1 wk and 2 wk stability time points.

Vehicle Characterization

Introduction

Intra-vaginal drug delivery is highly desirable for localized drug delivery to the female reproductive tract. However, the vaginal cavity, as a targeted delivery site, poses several challenges due to individual variability of physiological conditions such as a range of pH, varied microbiota, cervical mucus and cyclic changes during both menstruation and pregnancy. Mucosal drug delivery systems, in particular, have been limited by the mucus barriers. The CVM structure is an interwoven mesh network of mucus laden with bacteria, proteins, lipids, polysaccharides and ions. All of these aspects contribute towards mucus as a barrier towards pathogens [38]. The structure of mucus also changes over time due to hormonal fluctuations [39]. An effective formulation must work to overcome these physiological challenges through a designed formulation model that promotes absorption through the mucosal pores and into the epithelium. Therefore, three factors become crucial in vaginal drug delivery: the rheological and gel like properties of the vehicle, size and subsequent stability of particles passing through the pores (as previously outlined), and the osmolality of the formulation. Here, I was primarily focused on describing a gel like formulation that with thermosensitive properties to create an in-situ gel and promote extended release and absorption into the tissues and finding desirable strain characteristics that are similar to vaginal mucus which minimizes discomfort and secretions. Pluronic F127 has been used for thermosensitive polymers with reversible sol-gel transition behavior in aqueous solutions have been utilized for the vaginal delivery of a variety of drugs including 5-fluorouracil and metronidazole [40-41]. These gels provided a convenient and effective treatment. Furthermore, they have tunable properties and can easily be combined in a multi polymeric mixture for increased stability and viscoelasticity.

Methods

Pluronic F127 (12.6 kDa, 70% w/w PEO) was obtained from BASF (Mount Olive, NJ, USA) and used without further purification. Gels were prepared based on weight. Concentrations of Pluronic F127 are expressed by weight percentage (%, w/w). Pluronic polymer F127 was dissolved in deionized water and vortexed at room temperature. The solution was stored at 4 °C

overnight until the mixture was completely homogenous. This methodology was used to create ten F127 gels from 2-20% w/w.

Rheological measurements of polymer gels were performed on a strain-controlled rheometer, Anton Paar MC 302, using parallel plate configuration. The plates were set with 1 mm height during measurements. Temperature control was achieved using the lower plate and humidity was maintained using a fluid bath (DI water) surrounding the outer cylinder with a solvent trap. The 8mm plate geometry was loaded with the polymer solutions at 4 °C, in order to ensure that they were in the liquid state.

The instrument was used in the oscillatory mode, in which the plate geometry is rotated sinusoidally at a given frequency. Amplitude sweeps were conducted at shear strain from 0.1 to 1% strain. Frequency was measured at 0.1 to 100 rad/s. Temperature sweep measurements were performed at a constant heating rate of 1 °C/min until 50 °C. The frequency dependence of the complex modulus was determined between 0.01 and 100 rad/s. The sol-gel transition temperature of F127 gels was determined from an oscillatory shear temperature sweep at 1 rad/s. The sol-gel transition temperature was measured for all the gels. Finally, time sweeps measurements were performed at 25 °C and 37 °C for 10 mins.

In vivo tests of *in-situ* gelation and distribution were conducted using fluorescent F127 gels. The gels were prepared with 5% w/w Alex Fluor labelled florescent F127 at an absorbance of 568 and 95% w/w F127. The fluorescent gels were prepared by mass and mixed with deionized water at 6, 8, 10, 12, 14, 16 and 18% w/w.

Female CF-1 mice of 6-8 weeks were sourced from Harlan (Indianapolis, IN). Housing and handling were performed in accordance to guidelines and experimental protocols as approved by the Johns Hopkins Animal Care and Use Committee. Prior to vaginal administration, mice were injected subcutaneously with 2.5 mg of medroxyprogesterone acetate [Depo-Provera (depo); Pfizer, NY, USA] in 100 μL of phosphate buffered saline (PBS) 7 days prior to experiments. Mice were anaesthetized with isoflurane and vaginally dosed with 20 μL or 50 μL of fluorescent F127. The mice were allowed to wake for 15 minutes. Vaginal swabs were taken from each

mouse and the gelation of the F127 was visually observed and recorded. Mice were then euthanized through cervical dislocation. The vagina, cervix and proximal uterine horns were excised. The tissues collected from mice treated vaginally with were flash frozen in Tissue-Tek O.C.T. (Sakura Finetex, USA) using liquid nitrogen vapor. All tissue samples were stored at -80 °C until further analysis.

Tissue sections were cut at 10 μm thickness and mounted on Superfrost Plus Microscope Slides (Fisher Scientific). Following cryogenic sectioning, slides were fixed in 10% w/v formalin solution, neutral buffered (Sigma Aldrich) for 10 min followed by two washes with PBS. Slides were then incubated in 1X TBS for 5 min followed by a PBS wash. ProLong Gold antifade mountant with DAPI (Life Technologies) was applied directly to samples. Slides were imaged using Confocal microscopy.

Results and Discussion

Rheological Properties of Pure F127 Gels

Creating a vaginally compatible hydrogel requires understanding of the viscoelastic properties of the gel. Composition and concentration of gels can strongly affect rheology and lead to drastically different behavior. An oscillatory measurement system was employed because of its ability to measure both the elastic and viscous components. In order to measure the linear viscoelastic properties, we must find the linear viscoelastic region. The region was determined by measuring the storage (G') and loss (G") modulus as a function of the strain amplitude using a 20% w/w gel. The amplitude sweep was measured between 0.0001 and 2% strain at 1 rad/s. From Figure 6 A, I observed that G' and G" are independent of strain amplitude up to 1.8. Therefore, a strain amplitude of 1.0 at 1 rad/s shear rate was used for all the subsequent measurements to remain in the linear region and maintain the internal structure of the gel during dynamic measurements.

An ideal gel system would have rheological properties that increase the ease of application and the dispersion of gel across the vaginal canal. The gel should possess sufficiently viscous behavior that leads to retention in the mucus and offer elastic structure to facilitate sustained

release. Therefore, an *in-situ* gel that becomes physically entangled as temperature increases would improve application and have desirable viscoelastic characteristics. As early as 1945, Clift et al studied the rheological properties of vaginal mucus noting that flow-elasticity, plasticity and tack undergo significant changes throughout the menstrual cycle [42]. In 2003, Yoshida et al characterized human cervical mucus viscoelastic properties using various rheometric setups [43]. We also know based on ongoing pregnancy study that the properties of the CVM change as pregnancy progresses and the cervix ripens. Due to the high degree of variability in the CVM alone, without considering the effect of clearance rates and ambulation. Therefore, there is no single ideal viscosity value. As gels are non-Newtonian I determined a range of viscosities as a function of shear rate. Gels will experience a loss in viscosity at high shear rates if they have shear thinning behavior, as in Figure 6 D. The two-plate model provides a mathematical model for viscosity, where the lower plate is fixed and the upper plate drifts over the gel subjecting it to stress parallel to the upper surface. Viscosity on the Anton Paar system is based on 2 essential criteria:

1. fluid has contact with both plates through adhesive forces;
2. flow is laminar and turbulence is negligible.

Correct loading of gel into the plate system reduces the chances of error and subsequent incorrect calculations. Shear stress is first calculated based on force per square unit area (Equation 2). Shear rate is then measured from the velocity (v) as rotational speed at various points, the shear gap (h) is known and can be used to find the shear rate (Equation 3). The viscosity is finally found by shear stress over the shear rate as per Newton's law of viscosity (Equation 4).

$$\tau = \frac{F}{A}[=]\frac{N}{m^2} \quad \text{Equation 2}$$

$$\dot{\gamma} = (D =)\frac{v}{h}[=]s^{-1} \quad \text{Equation 3}$$

$$\tau = \eta(\dot{\gamma}) \rightarrow \eta = \frac{\tau}{\dot{\gamma}} \quad \text{Equation 4}$$

Overall for all concentrations of F127 as the shear rate increases the viscosity decreases. Gels of 16% up to 20% w/w start at a very high viscosity and decrease by almost two orders of magnitude at high shear rates at 50 s^{-1}. 14% w/w F127 gel had a viscosity of 49.0 Pa s at a shear rate of 0.5 s^{-1} and decreased to 2.2 Pa s, indicating shear thinning behavior for all gels from 10 to

20% w/w. This pseudo-plastic flow behavior is characterized by a decreasing viscosity with increasing shear rate, thus as shear rate increases molecules in the hydrogel undergo disentanglement and the individual polymers have less flow resistance. Therefore, the gel would be suitable for vaginal delivery and still provide coating across the mucus at higher shear rates from the delivery medium.

Since materials can display a mixture of both viscous and elastic behavior when sheared, viscoelastic behavior needs to be measured. Particular attention is paid to the elastic portion since it becomes more pronounced with faster movements (higher shear or frequency) and at lower temperatures. Under these two conditions the molecular network of a gel becomes more rigid. In contrast, higher temperatures and/or less motion allows gels to be more mobile and flexible. Thus, viscosity alone is insufficient as a metric for materials characterization as elastic effects can greatly determine the performance of a gel. Therefore, in addition to viscosity measurements and amplitude sweeps, frequency, temperature and time sweeps were conducted. Frequency tests indicated the viscoelastic behavior of the gel and provide a quantitative measure of the fluid dynamics of a given gel. The two-plate model allows us to measure shear stress (Equation 2) and shear strain (Equation 3). We can calculate the shear modulus:

$G = \frac{\tau}{\gamma}[=]Pa$……..Equation 5

The oscillatory system allows a motor to move the top plate with a deflection of the upper plate at angles 0°, 90°, 180°, 270° and 360°. A maximum deflection and oscillatory period are evaluated as strain or deformation. The parameters for oscillatory tests present as a sine curve and from this we can find a maximum deflection or amplitude and its oscillatory period or frequency. This is where an amplitude determines the critical strain for a gel. Once this is found we can use the shear stress (measured by the counter force that acts on the lower plate). We obtain two resulting sinusoidal curves that have a phase shift δ, if viscoelastic. The phase shift for fluids are $90° \geq \delta \geq 45°$, while at rest. When $\delta = 0°$ the material has ideally elastic deformation behavior and when $\delta = 90°$ the material has ideally viscous flow behavior. Solids and gel like states have a phase shift of $45° \geq \delta \geq 0°$, at rest. Storage moduli (G') represents the elastic portion which is quasi indicative of solid-state behavior. The loss modulus (G") characterizes the viscous component. Internal friction from the components of a flowing fluid causes viscous behavior. The friction produces frictional heat and transforms deformation energy

to heat energy. Part of this energy is absorbed by the sample and this loss of energy is what defines the loss modulus. The storage modulus is defined by the elastic portion of energy that is stored in the deformed material.

From our samples of F127 gels, the gels containing 12% w/w F127 had a higher loss to storage moduli ratio (G'>G"), meaning they were more fluid. And thus, could have weaker bonds between individual molecules. While gels greater than 14% w/w (up to 20% w/w) formed gels as indicated by the higher elastic modulus, indicating stronger physically bonding between molecules. Non-destructive deformation of the gel was seen at higher frequencies >30 rad/s for 16, 18 and 20 % w/w gels. Gels below 14% w/w experienced inconsistent frequency behavior in the elastic modulus around the frequency range.

The sol-gel transition temperature, and the elastic modulus of the F127 solutions were found to depend on the concentration of the polymer. Above the gel transition, the elastic modulus of the F127 gel plateaus with an increase in temperature. At lower F127 concentrations of 2%, 4% and 6% w/w no definitive gelling could be determined, likely due to the higher water content. At gels between 8-12% w/w, gels achieved a sol-gel transition point between 27-30 °C before deforming.

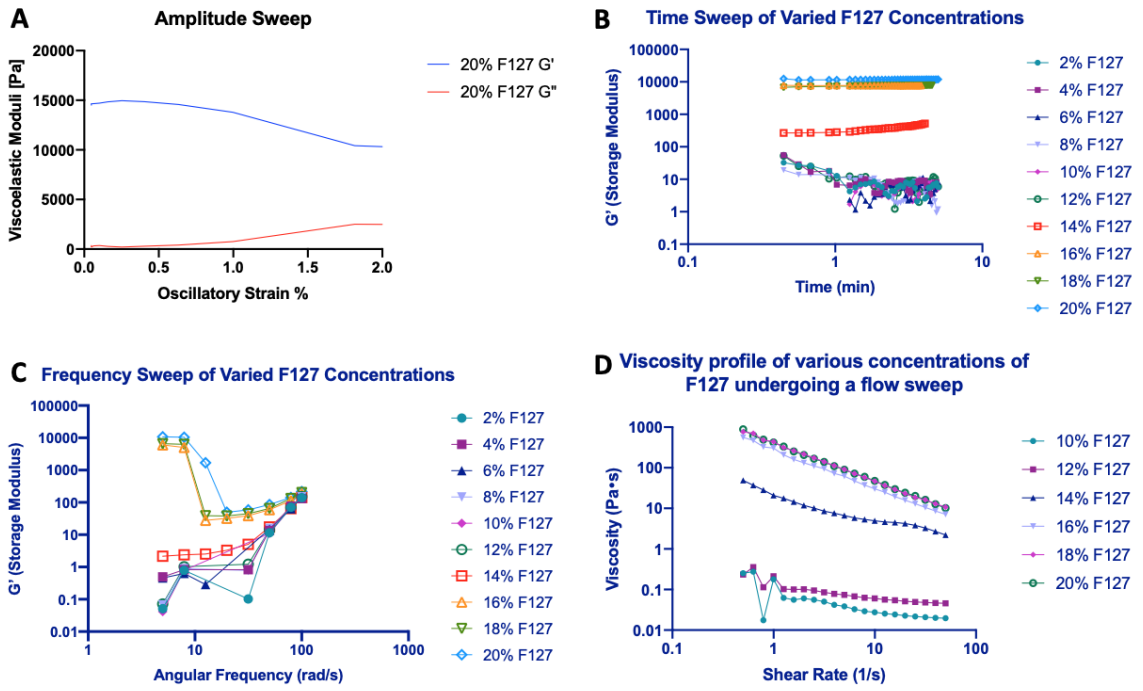


Figure 6. A. Amplitude sweep at the highest used concentration of F127 (20% w/w) indicated independent storage and loss moduli from 0 to 1.8% strain. B. Time sweeps showed stability of gels greater than 14% w/w over 600 sec time period. C. Storage moduli of various gels in frequency sweeps indicate varied fluid-gel dynamics that may correlate to F127 concentration. D. Viscosity profile of F127 from 10 to 20 % w/w indicates shear thinning behavior for F127 hydrogels. Concentrations of ≥14 % w/w had more consistent pseudo plastic behavior due to higher viscosity and lower water content.

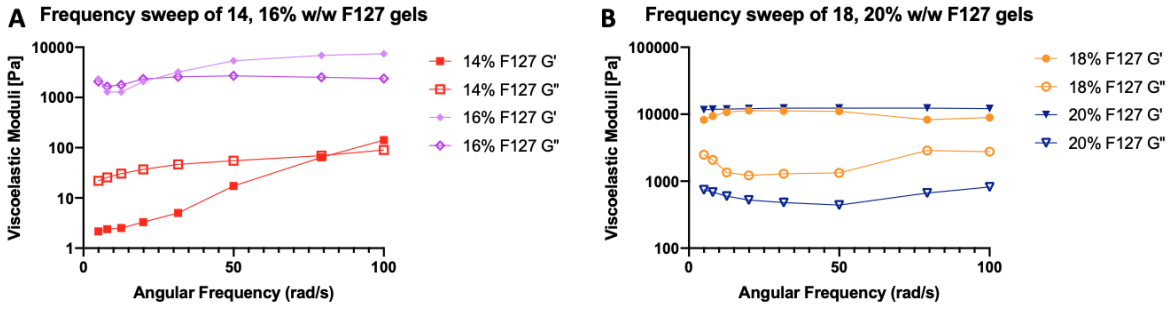


Figure 7. A. At lower concentrations of F127 (14, 16% w/w) that still maintain gel like dynamics, gel is predominately in a fluid-like state at lower frequencies G"›G' even at lower strains. B. At higher concentrations of F127 (18, 20% w/w) gels are in a solid or gel like state as G'›G"

In vivo Gelation of Fluorescent F127 Gel

The gelation of F127 hydrogels was in vivo confirmed by vaginally dosing mice. Gels of >10% w/w F127 converts to the gel phase at 37 °C when inserted vaginally. As seen in Figure 8, F127

aqueous solutions had a viscous gel like consistency on the vaginal swabs. Visual observation showed that viscosity increased with increasing concentration of F127. Gels of 6 and 8% w/w did not gel after the dosing procedure and remained liquid both intravaginally and on the swab after 15 minutes based on visual observation. Therefore, gels of higher concentrations, 10-20 % w/w, are more effectively become a gel *in-situ* and thus, are suited for vaginal dosing in mice (Figure 8).

In terms of tissue distribution from confocal imaging, our hypothesis was the F127 hydrogel would along the surface area of the tissues and throughout the epithelia due to the nanoparticle size and the had better penetration through the reproductive tract. Gels of 16% had the greatest permeation through the tissues (Figure 9).

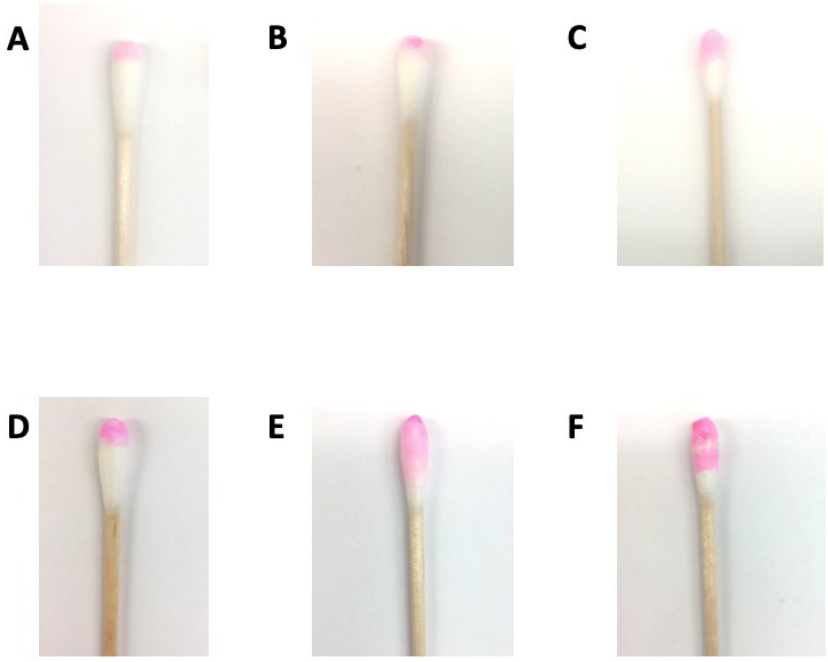


Figure 8. Swabs of fluorescently labelled F127 in aqueous suspensions at various concentrations 6, 8, 10, 12, 14 and 16 % w/w, A-F respectively. As the concentration increases a more viscous gel like consistency is achieved once the mice were vaginally swabbed.

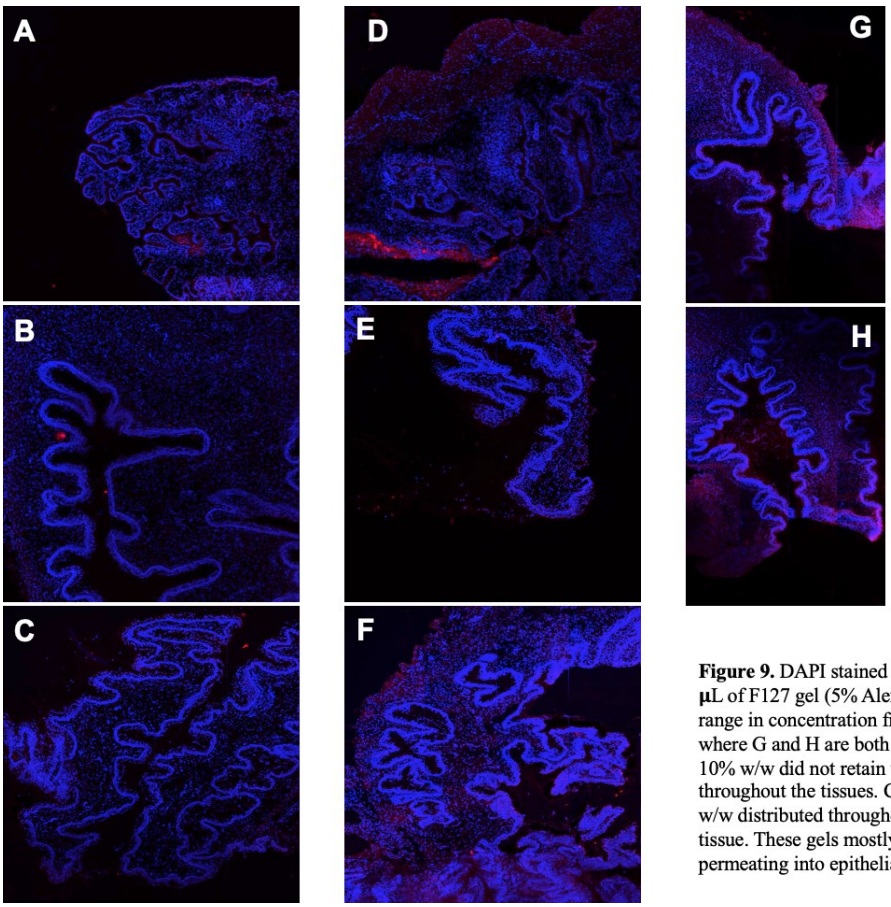


Figure 9. DAPI stained vaginal and cervical tissues with 20 **µ**L of F127 gel (5% Alexa Fluor F127 and 95% F127). Gels range in concentration from 6% w/w to 18 % w/w (A-H, where G and H are both 18% w/w). Tissues from 6% w/w to 10% w/w did not retain the gel well and have distribution throughout the tissues. Concentrations of 12% w/w to 18% w/w distributed throughout vaginal epithelia and to cervical tissue. These gels mostly surround the tissue and can be seen permeating into epithelia at 12, 16 and 18% w/w F127 gels.

Hypotonic Gel

Introduction

The rheological and gelation properties of Pluronic solutions can be impacted by the presence of various additive such as polymeric stabilizers and drug molecules. Therefore, when designing a hydrogel, it is imperative to test the gel's physical properties with these additives. Most traditional polymer gels are formed through a swollen network of covalently crosslinked polymer chains. However, the Pluronic triblock copolymer is held by reversible entanglements of the coronae of neighboring micelles. The reversible nature of coronae entanglements is due to an absence of covalent crosslinking between micelles. The structure of these micelles into layers causes adaptive laminar shear flow and consequentially is responsible for the exceptional viscoelastic properties of Pluronic gel systems. Previous studies have shown that the viscoelastic properties are extremely resilient when subjected to shear forces. Therefore, the properties of F127 gels as previously demonstrated are dynamic and vary with shear rate and concentration. However, the organization of the physically linked micelles is changed with supplemental additives in the solution. Thus, it is important to further characterize the rheology of hydrogels loaded with nanoparticles or stabilizers.

Hoffman, Ackerson and Loose pioneered work on shear induced transitions in suspensions of charged particles and subsequently the field studying the structural properties of colloidal crystals under shear has expanded significantly [43-44]. Recently, Jiang has shown that selective triblock copolyethers in an aqueous solution can lead to the formation of micelles with spherical core-shell geometry [45]. Dilute solutions typically contain discrete micelles, whereas highly concentrated solutions form highly ordered micelles. The packing order of these structures and their coronae can be influenced by environmental conditions such as higher shear and therefore, it is crucial to determine the physical limitations of each gel with NC and measure their effectiveness within the vaginal microenvironment.

Methods

Crinone® 8% gel (Allergan, Inc.) was obtained from Johns Hopkins Pharmacy. Pluronic® F127 was obtained by BASF (Ludwigshafen, Germany). Progesterone (P8783-5G) was sourced from

Sigma-Aldrich. Pure F127 gels at a concentration of 14, 16, 18, 20% w/w were formulated as previously described. Solutions were kept at 4 °C until their next use. 8% progesterone nanoparticles were created using wet milling. Particle hydrodynamic diameter, polydispersity index (PDI), and surface charge (ζ-potential) of the progesterone NS formulations were measured using a Malvern Zetasizer Nano ZS (173° scattering angle). The NS were diluted 1:1000 in 10 mM NaCl (pH 7) for ζ-potential measurements. The final NS formulation contained 80 mg/ml progesterone (8%) in 2% w/w F127 and 2% w/w F68. Batches were milled for 10 h until the final average size of 260 nm was obtained. NS was separated from beads and lyophilized for 72 h. The final dry NS mass was then reconstituted with either the 14, 16, 18 or 20% w/w F127 vehicle gel, forming a progesterone hydrogel.

Physical Gel Measurements

Hydrogels were then tested for physical characteristics including size, ζ-potential, osmolality and rheometry. Size, ζ-potential and polydispersity index measurements were carried out as previously described using dynamic light scattering on a Malvern Zetasizer Nano ZS. The tonicity of Crinone® and hydrogels was measured as previously described. Osmolality was measured using Wescor EliTechGroup Vapor vapor pressure Osmometer. The device was calibrated and 10 µL of undiluted hydrogel was measured. Additionally, the pH of F127 at various concentrations, Crinone® and progesterone loaded gels were measured using a Mettler Toledo pH meter. Rheological measurements of polymer gels were performed on a strain-controlled rheometer, Anton Paar MC302, using parallel plate configuration. The plates were set with a distance of 1 mm during measurements. Temperature control was achieved using the lower plate and humidity was maintained using a fluid bath (DI water) surrounding the outer cylinder with a solvent trap. The 8 mm plate geometry was loaded with the polymer solutions at 4 °C, in order to ensure that they were in the liquid state. The instrument was used in the oscillatory mode. Amplitude, frequency, temperature and viscosity measurements were conducted as previously described.

RU486 Efficacy

Timed pregnant 6-8-week-old female CD-1 mice were ordered from the Charles River Laboratories (Wilmington, MA). Pregnant mice were delivered to the Johns Hopkins animal facility between 8 to 10 days of gestation (E8-E10). The mice were acclimated to a reverse light cycle room, with a dark period from 10 am – 10 pm) prior to conducting any procedures. All procedures began on E15 out of a total 19.6 day gestation period (range of 19-20 days). All procedures were approved by the Johns Hopkins Animal Care and Use Committee. I performed efficacy tests using a RU486 progesterone antagonist (Sigma-Aldrich M8046). RU486 was dissolved in dimethlysulfoxide (Sigma-Aldrich) at a concentration of 0.5 mg/mL. Pregnant mice were dosed subcutaneously in the scruff of the neck with 25 μg of RU486 in 100 μL on the morning of E15. Four distinct groups were maintained: control with no vaginal dosing, nanocrystal solution, NC hydrogel and Crinone®. The NC, hydrogel and Crinone® were all vaginally dosed. Two sets of efficacy experiments were performed with all four groups where the vaginal therapies were dosed with a total volume of 50 μL. Mice receiving the vaginal progesterone treatment were given 8 mg of progesterone using a 50 μL Wiretrol (Drummond Scientific) in the form of 50 μL of hydrogel, Crinone® gel or 50uL of the NS formulation. The second set only compared NC hydrogel with Crinone® and RU486 antagonist alone, dosed at a volume of 20 μL of either nanoparticle loaded hydrogel or Crinone® gel. NS solutions were prepared up to 72 h prior to animal dosing. Treatment began at the time of RU486 injection and continued daily each morning until E18 or until PTB occurred. Mice did not receive further treatment if they delivered preterm, mice that remained pregnant throughout treatment received up to four doses. Animals were counted as delivered to term if the birth occurred after the night of E18 (E18.75 >day). Upon analysis, statistical analysis of groups of three or more was conducted using the Log-rank test and the Fisher's exact method for survival analysis in GraphPad Prism 8.

Results and Discussion

Rheological Properties

Gel performance *in vivo* is strongly linked to the rheological properties of the gel. Spreading, retention and drug release behavior can be predicted based on rheological behavior. Comprehensive rheological characterization of drug delivery systems, as platforms and in mixed form, can provide drastically different results. In order to measure the linear viscoelastic

properties, we must find the linear viscoelastic region. The region was determined by measuring the storage (G') and loss (G") modulus as a function of the strain amplitude using a 20% w/w gel. The amplitude sweep was measured between 0.0001 and 1 at 1 rad/s. From Figure 10 A, I observed that G' and G" are independent of strain amplitude up to 0.01%. Therefore, a strain amplitude of 0.001 at 1 rad/s shear rate was used for all the subsequent measurements to remain in the linear region and maintain the internal structure of the gel during dynamic measurements.

In terms of viscosity, nanoparticle loaded gels at 16, 18 and 20 % w/w F127 had shear thinning behavior similar to F127 alone (Figure 10 B). However, 14 % w/w gel experienced a less discernable viscous behavior. 14 % w/w gel maintained its viscosity at lower strain rates before decreasing in viscosity at higher shear rates. While the viscosity of 20 % gel based on manual handling was difficult to use at room temperature. 16 and 18 % w/w presently are the best candidates for hydrogel in vivo delivery. Additionally, frequency sweeps were conducted with all two of the previous nanoparticles, selected based on the most favorable size, ζ-potential and stability data: 2% F127 coated particles and 2% F127 with 2% F68 coated nanoparticles. Both were mixed with 16% and 18% F127 gels based on weight. Both sets of particles and gels demonstrated fluid like behavior at low frequencies (G">G') and solid like behavior at higher frequencies (G'>G"). Both gels and nanoparticles experience irregular viscoelastic behavior at 40 rad/s, the cause of which has not been determined.

The temperature dependence of rheological properties of the gels was studied at a range of 20 °C to 50 °C. In Figure 11, I plotted the storage modulus as a function of temperature. For all 14, 16 and 18% w/w hydrogels the storage modulus is low at lower temperatures but increases significantly with temperature, corresponding to the formation of gel. At the end of the sol-gel transition, the storage modulus becomes independent of the temperature. This is seen by the plateau of G'. At the higher concentration of 20 % w/w at 20 °C the gel has already formed therefore, in the rheological measurements the gels have already reached the end of the sol-gel transition and G' begins to plateau around 25 °C (Figure 11**).** Furthermore, when comparing four formulations (2% F127 NP in 16 % F127 gel (1.16), 2% F127 & 2% F68 in 16 % F127 gel (3.16), 2% F127 NP in 18 % F127 gel (1.18) and 2% F127 & 2% F68 in 18 % F127 gel (3.18)) I found that both gels of 2% F127 and 2% F68 coated NP had a distinct crosslinking point from a

predominately fluid state (G”>G’) to a solid state (G’>G”). Formulation 3.16 as a lower concentration of F127 (16% w/w) experienced gelling at 29.4 °C while formulation 3.18 gelled at 23.0 °C. Both 2% F127 NP gels had a greater storage modulus to loss modulus at all temperatures from 20-50 °C (G’>G”) indicating a more solid state. The behavior of the 2% F127 and 2% F68 coated NP is desired for an *in vivo* thermogelling drug system and suggests more tunable behavior than the 2% F127 NP gels. Thus 16% and 18% w/w F127 gels with 2% F127 and 2% F68 NP would be suitable to move forward with *in vivo* application.

Based on these results there is some but limited impact from the addition of 2% F127 and 2% F68 coated nanoparticles on the physical crosslinking and viscoelastic properties of the progesterone hydrogel. As determined by the amplitude sweep the linear viscoelastic region was defined from 0 to 1% strain. The structures of the 2% F127 and 2% F68 coated NP 16% F127 hydrogel (herein referred to as NP gel), Crinone® and nanocrystal were further characterized using a frequency sweep below the critical strain γ_c. In frequency sweeps at 0.1% strain the hydrogel shows a more fluid-like behavior at high strain amplitudes where G”>G’ whereas they are more solid-like at low strains (G’>G”). The nanocrystal had inconsistent properties where both moduli oscillate without any dependence on the frequency (Figure 12). Overall, the hydrogel demonstrated a higher elastic modulus in comparison to Crinone®, possibly improving distribution throughout the CV tract. Consistent shear thinning behavior similar to Crinone® and substantially improved from nanocrystal alone. Gels at 18-20% w/w at a higher viscosity than Crinone®, however gels of 14-16% w/w had a similar viscosity and displayed desired shear thinning profiles without substantial viscosity changes (not shown).

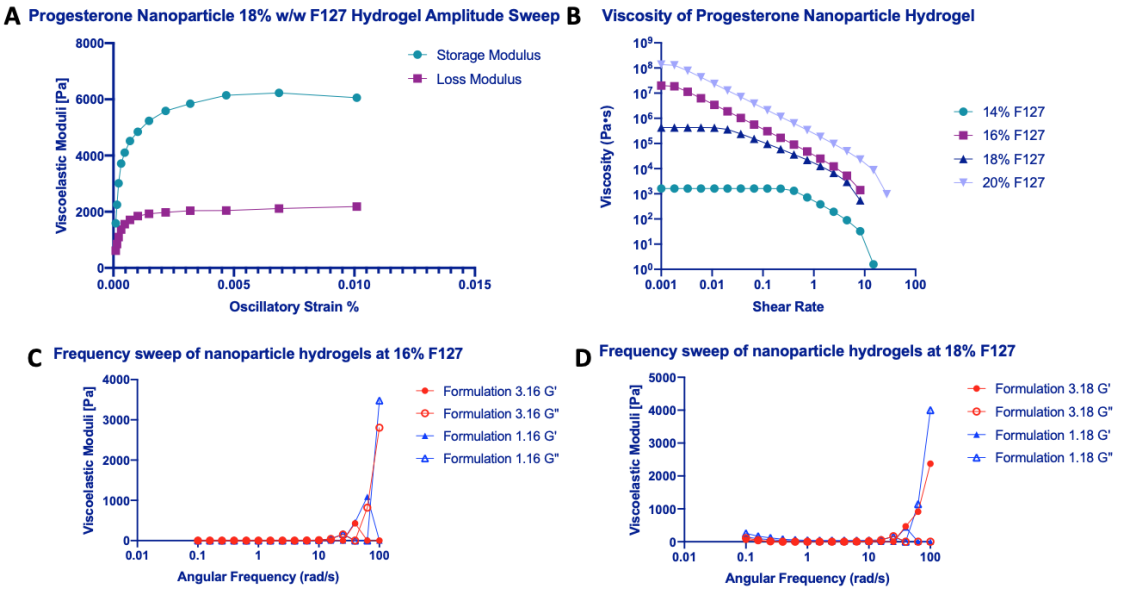


Figure 10. A. Amplitude sweep of 18% w/w F127 hydrogel loaded with 2% F127 and 2% F68 coated progesterone nanoparticles showed moduli independence at strains up to 0.01%. B. Viscosities of 2% F127 and 2% F68 coated progesterone nanoparticles in varied concentrations of F127 gel. 16 and 18 % w/w gels demonstrated viscosities comparable to previous gels with nanoparticle and desired shear thinning behavior. Frequency sweeps comparing two types of formulations 2% F127 NP in 16% w/w gel v 2% F127 and 2% F68 in 16% w/w gel (C.) and 2% F127 NP in 18% w/w gel v 2% F127 and 2% F68 in 18% w/w gel (D.)

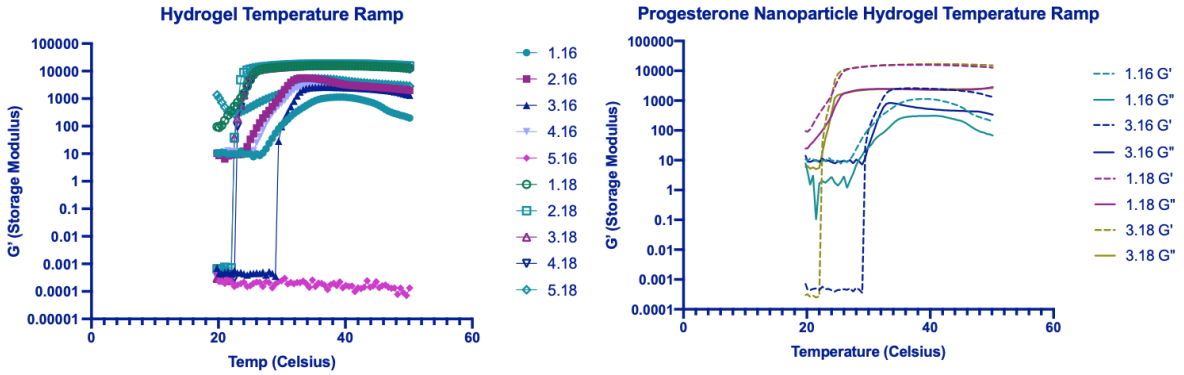


Figure 11. Storage modulus (G') of various nanoparticle formulations mixed into 16% and 18% w/w F127 gels. Most gels experienced gelation between 20 and 25ºC, expect for 4% F127 NP in 16% gel (5.16) which never underwent physical crosslinking. B. When comparing four gel formulations (2% F127 NP in 16 % F127 gel (1.16), 2% F127 & 2% F68 in 16 % F127 gel (3.16), 2% F127 NP in 18 % F127 gel (1.18) and 2% F127 & 2% F68 in 18 % F127 gel (3.18)), only the 2% F127 & 2% F68 NP gels had definitive crosslinking from a fluid state (G">G') to a solid state (G'>G").

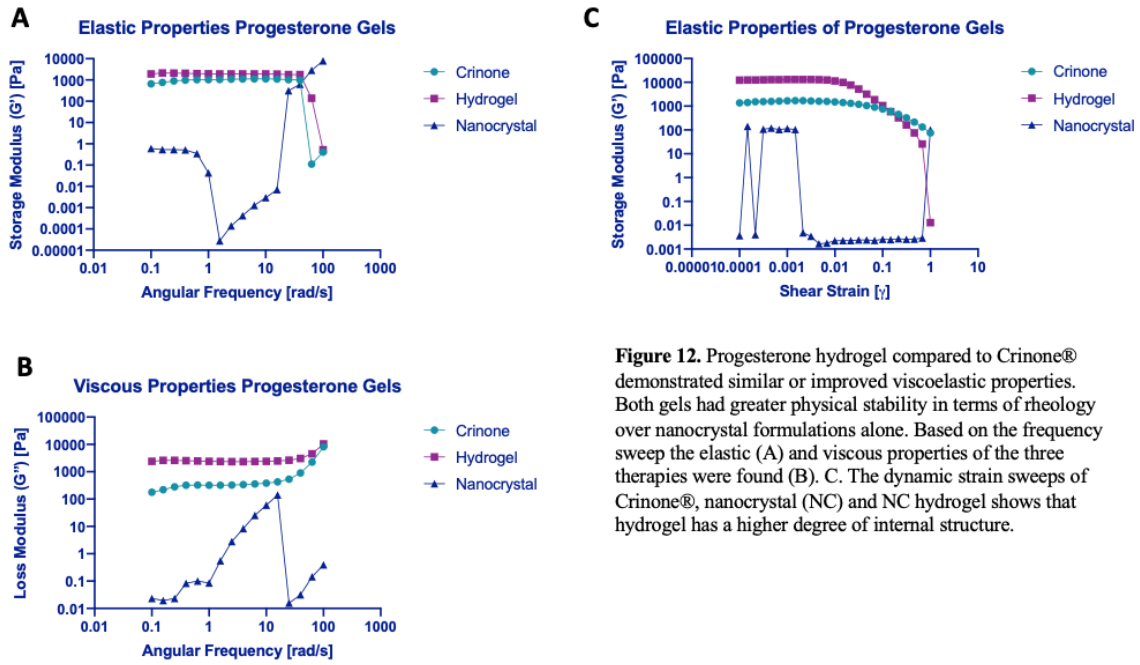


Figure 12. Progesterone hydrogel compared to Crinone® demonstrated similar or improved viscoelastic properties. Both gels had greater physical stability in terms of rheology over nanocrystal formulations alone. Based on the frequency sweep the elastic (A) and viscous properties of the three therapies were found (B). C. The dynamic strain sweeps of Crinone®, nanocrystal (NC) and NC hydrogel shows that hydrogel has a higher degree of internal structure.

Other Physical Properties

It has been found that the pH of Crinone® (3.2) was similar to the vaginal microenvironment which ranges from 3.5 ± 0.3 [46]. The mucoinert progesterone NPs alone was 7.2 and NPs in F127 ranged from 6.88 to 7.02 as seen in Table 2. In the future adding excipients such as carbonate derivatives or lactic acid to reduce the pH might improve the in vivo performance of the gel. We were also able to determine the tonicities of NP gel and Crinone®, 144 and 1560 mOsm/kg, respectively. While our NP gel is hypotonic this is an improvement from Crinone® which is hypertonic. In this case the osmolarity of our gel could be optimized to improve the net flow of fluid into the vaginal epithelia.

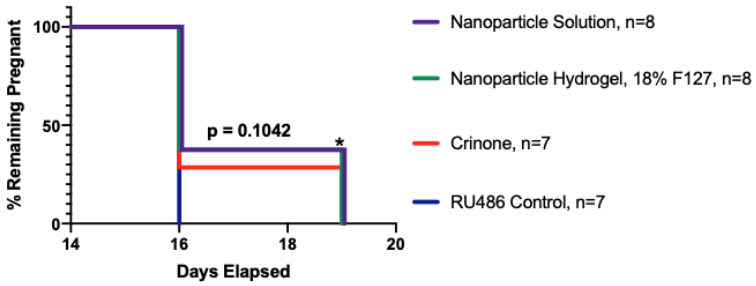
	Type of Particle			
pH	1	2	3	4
Gel % w/w	2% F127	2% F127 + 2% TPGS	2% F127 + 2% F68	2% F127 + 2% HS15
14	6.88	6.93	6.93	6.92
16	6.93	6.96	6.96	6.93
18	6.93	6.98	7.02	6.92
20	6.99	7.02	6.99	6.88
Crinone®	3.24			

Table 2: Average pH of various progesterone gel formulations. The pH of a market approved therapy Crinone® is drastically lower in comparison to formulated NP based F127 hydrogels.

RU486 Efficacy

RU486, or Mifepristone, is a well-studied competitive progesterone receptor antagonist, which provides us with a functional progesterone withdrawal model. As reported by Hoang et al a dose of 25 μg RU486 via subcutaneous injection is resulted in preterm birth in 85% of control animals within 24 h [36]. 50 μg RU486 in 100 μL was dosed to all mice. All control mice gave preterm within 24 h at E16. Here we observed that 8 % progesterone delivered at a volume of 50 μL could oppose the effects of RU486 in mice. This was observed in both in the nanocrystal formulation as well as the 18 % w/w F127 progesterone loaded gel. At E19 both progesterone nanocrystal and progesterone hydrogel had 37.5% of mice remain pregnant. Comparatively, Crinone® was less effective with 28.6% of pregnancies maintained at E19 (Figure 11 A). At a lower volume of 20 μL of gel vaginal therapies at 8 % progesterone, the gel nanoparticle was less effective than Crinone®, with only one third of mice remaining pregnant at E19. Crinone® gel was able to prevent over 50% of preterm births. While neither result was statistically significant, the variation in these results could be due to a number of conditions including the pH of the gel and the decrease in gel concentration from 18% to 16%. Furthermore, both domestic and wild mice seasonally experience changes in breeding and the gestation stages as reported by Drickamer et al [47]. The seasonal variation can result in reduced pup size, decreased viability, lower litter sizes over winter months in comparison to the fall, spring and summer. It is hypothesized that shifting rates of breeding and pregnancy could be a natural mechanism to account for resource availability and climate conditions. Decreased efficacy in the 20 μL dosed progesterone gel RU486 antagonist experiment may be partially due to the overall diminished viability of mice pregnancies over the winter.

A **PTB prevention with progesterone vaginal therapies**



* nanoparticle solution and nanoparticle hydrogel have overlapping results, 37.5% survival at E19

B **PTB prevention with progesterone vaginal gel therapies**

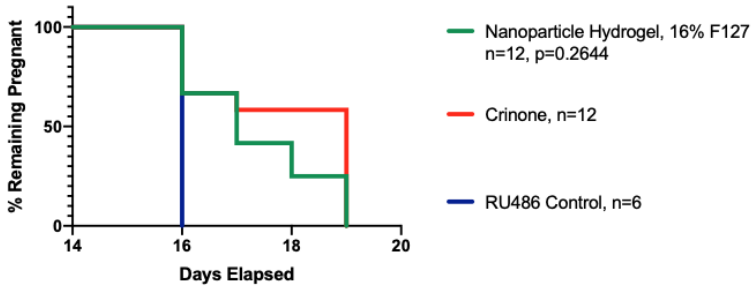


Figure 13. A. PTB prevention rate of mice vaginally dosed at 50**µ**L of 8% progesterone therapies, 2% coated F127 nanocrystal (NC), NC in 18% hydrogel and Crinone®. B. In gel therapies dosed at 20**µ**L NC had a lower PTB prevention at E19 than Crinone®, however not statistically significant.

Conclusion

Overall, I was able to define the properties of a potential candidate hydrogel for vaginal drug delivery. From more than 90 unique combinations of milled progesterone loaded nanoparticles I found five with a near neutral ζ-potential, with a hydrodynamic diameter of less than 300 nm and a PDI below 0.25.

As a stand-alone gel, F127 was characterized for physical properties. Both the 2% F127 progesterone loaded nanocrystals and the 2% F127 progesterone nanocrystals with additional 2% w/w Pluronic F68 stabilizer demonstrated the greatest stability. Furthermore, I found that solutions of 14-20% (w/w) F127 demonstrated desirable rheological properties in terms of viscosity, gelation, shear thinning behavior and viscoelastic dynamics. Pluronic F127 based hydrogel also had similar or improved elastic properties compared to Crinone® and to the nanoparticle suspension. I found that both the 16 and 18% w/w progesterone hydrogels were hypotonic and demonstrated Crinone® had toxicity in mice likely due to the hypertonicity. However, the pH of the hydrogel could be optimized to improve *in vivo* efficacy. I was able to successfully create a gel formulation that would be safer for chronic daily use and showed similar efficacy to Crinone®.

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Curriculum Vitae

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EDUCATION

MSE Johns Hopkins University, Chemical and Biomolecular Engineering May 2019
Thesis: "The Development of Thermoreversible Progesterone-Loaded Hydrogels for the Prevention of Preterm Birth"
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BS Johns Hopkins University, Chemical and Biomolecular Engineering May 2016
Molecular and Cellular Bioengineering Concentration

HONORS AND AWARDS

Tocris Bioscience Scholarship	2018
Chemical and Biomolecular Engineering Master Scholarship	2018
GEMS Student Travel Award	2018
Dean's Fellowship	2017
Institute for Nanobiotechnology IRES	2015
Provost Undergraduate Research Award	2012
Mattie and George Newman Memorial Scholarship	2013
Terence Flannery Memorial Annual Scholarship	2013
American Foreign Service Association Merit Award	2012
Marcia Martin and Brockman M. Moore Memorial Scholarship	2012

RESEARCH EXPERIENCE

Johns Hopkins University, George Mason University, La Sierra Bolivia 2018 to 2019
Advisor: Dr. Amanda Debes

- Developed a nanocage hydrogel to target tuberculosis antigens *in vitro* from pediatric urine samples while at George Mason University
- Optimized reactive blue and reactive black dot blots for the detection of tuberculosis antigens and subsequent development of a point-of-care lateral flow assay
- Collected and analyzed clinical samples from suspected tuberculosis cases in Bolivia

Thesis, Johns Hopkins University, Baltimore MD 2017 to 2019
Advisor: Dr. Laura Ensign

- Developed progesterone hydrogel formulations that would rapidly penetrate cervicovaginal mucus in pregnant women

- Tested the mechanism of inflammation in mouse models through supplementation with progesterone and trichostatin A
- Characterized the structural and barrier properties of women's cervicovaginal mucus during pregnancy

US Centers for Disease Control & Prevention, Yaoundé Cameroon 2017
HIV Treatment Associate

- Conducted hospital visits with implementing partners to ensure quality of patient care and gathered baseline data for key indicators to enable measurement of progress
- Analyzed patient data using Excel and MATLAB to find errors in patient treatment and inefficiencies in care sites and created a linkage model for the FY18 country operation plan
- Trained clinical staff in the delivery of key population services and developed standard operating procedures on HIV/AIDS treatment

Sanaria Inc. & Protein Potential, Rockville MD 2016 to 2017
Research Associate

- Led a team of researchers and scientists in the formulation, manufacture and analysis of a Phase 1 foam dried drug to treat anthrax, typhoid and shigellosis
- Manufactured a novel vaccine for malaria in a GMP facility using aseptic technique, cGMP, GCP, GDP for Phase 2 and 3 clinical trials
- Developed a new method of cryopreservation and long-term stability for live vaccines through flash freezing

Interuniversity Microelectronics Center (imec), Leuven Belgium 2015
Engineering Research Intern

- Characterized the metastatic process of LNCaP and MDA-MB-231 cells using lens free imaging
- Fabricated microfluidic devices in an ISO 3 cleanroom using sterile technique for cancer mobility assays
- Developed an automated cell tracking method in MATLAB to process wound healing assays

Johns Hopkins University, Baltimore MD 2013 to 2016
Advisor: Dr. Konstantinos Konstantopoulos

- Awarded the Provost Undergraduate Research Award to determine breast cancer decision making in branched microchannels
- Tested the effect of anti-adhesion drugs on cancer cell migration in microfluidic chambers resembling vasculature
- Conducted experiments to understand the novel signaling mechanisms of cancer cells through physically confined microenvironments

TEACHING EXPERIENCE

Arbor Tutors, Baltimore MD May 2018 to present

Tutoring Instructor, SAT and GRE Mathematics

- Developed individualized syllabi and lesson plans for students
- Taught several students math topics including but not limited algebra, geometry, introductory calculus, statistics and probability

InnoWorks, Baltimore MD September 2012 to May 2015
Tutor, General Science & Mathematics

- Tutored students on a weekly basis on middle school math and science
- Developed coursework and relevant learning materials for a week-long summer camp for middle school students
- Taught students about basic science including simple physics (kinematics and optics), chemistry and biology

PUBLICATIONS

Journal Publications

Paul, C. Shea, D. Mahoney, M. **Laney, V.** Chai, A. W.-C. Hung, Konstantopoulos, K. *The Interplay of the Physical Microenvironment, Contact Guidance and Cell Signaling in Cell Decision Making* (2016) The FASEB Journal

Conference Proceedings

Laney, V. Zierden, H. Hernandez, N. Bensouda, S. Ensign, L. *Rheological Properties of Thermoreversible Nanoparticle Hydrogels for Vaginal Drug Delivery* (2018) Indianapolis, IN

Laney, V. Huang, H. Wu, Y. Jackson, J. Li, M. Gao, L. Xu, R. Chakravarty, S. Sim, KL. James, E. *Stabilization of the recombinant Ty21a-vectored vaccine, TyOraSs, against Shigellosis by foam drying* (2017) Washington DC

Laney, V. Paul, C. Chai, A. *MDA-MB-231 Breast Cancer Decision Making in Y-Shaped Microchannels* (2015) Baltimore, MD

Laney, V. Mathieu, E. *Analysis of LNCaP and MDA-MB-231 Motility Using Lens-Free Imaging* (2015) Leuven, Belgium

PROFESSIONAL TRAINING

Pharmacology Course, Foundation for Advanced Education in the Sciences, 2017

Aseptic Technique Workshop, Sanaria Inc., 2017

cGMP Certification, Sanaria Inc., 2017

PROFESSIONAL AFFILIATIONS

American Institute of Chemical Engineers
National Society of Black Engineers
Society of Women Engineers
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PROFESSIONAL SERVICE

Symposium Founder and Co-Organizer
Johns Hopkins Institute NanoBiotechnology Undergraduate Research Symposium

- "Frontiers of Medicine: Biological & Engineering Research Driving the State of Health Care" November 10, 2016
- "Innovations in Medicine, An Engineering and Biological Perspective" November 5, 2015

LANGUAGES

English: Native Language

Spanish: Advanced Listener, Advanced Speaker, Superior Reading and Writing

COMPUTER SKILLS

Programming: MATLAB

Applications: ASPEN, GraphPad